FINAL REPORT

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on the study of

THE RELATION OF AMINO ACIDS TO THE HEALING OF EXPERIMENTAL WOUNDS

under the supervision of

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When this project, on the effect of amino acids on the healing of experimental wounds, was first undertaken, the reports published on the subject of the healing of wounds were found to be exceptionally extensive. However, there were very few papers which approached the subject from the experimental point of view, most of them dealing with clinical material. Since many factors may affect the healing of wounds, only the results of work in which every factor is closely controlled, or at least equated by the use of rigorously matched groups of experimental animals, could be considered valid. A further difficulty inherent in this problem was the fact that the methods for measuring the rate of healing of wounds were not overly reliable and hence required that very large numbers of animals be used in order to obtain statistically valid results.

Of the work published on the healing of wounds, three main facts stood out and could be considered reliable in that they had been verified by many workers in the field. First, a negative nitrogen balance becomes evident after trauma; second, feeding a high protein diet results in an increased rate of healing; third, increased amounts of the amino acid, methionine in the diet results in an increased rate of healing. It should be noted that contributions to the metabolic picture during the healing of wounds were quite scanty. Before a real understanding of the processes involved in the healing of wounds could be developed, further knowledge of the metabolic reactions appearing after trauma would be required.

With this background, the work in my laboratory was undertaken. The first problem was to test the technique for measuring the rate of healing. It was found that modifications of my previously published method (Science: 1947: 105, 396) would give results which were statistically valid when groups of at least eight animals were employed. Using this technique, which involved the determination of tensile strength of wound tissue, the affect of a number of amino acids on the rate of healing was determined, under a number of different conditions. It was found that only methionine and cystine caused an increased rate of healing of the experimental wounds, while ethionine inhibited the rate of healing. The other

amino acids tested had no effect.

From these experiments it was concluded that the availability of the sulfur amino acids limited the healing process. Further experiments showed that a high protein diet increased the rate of healing, not because more protein or nitrogen was available, but because a high protein diet provided a greater supply of the sulfur amino acids. During the course of these experiments, it was noted that there was a retention of sulfur, even though a negative nitrogen balance existed. It was then concluded that the tissue proteins were broken down in order to make more sulfur amino acid molecules available for the healing processes. The other, unneeded amino acids are excreted, resulting in the negative nitrogen balance.

The source of the conserved sulfur amino acids and the purpose for which they are conserved was studied using radioactive-sulfur labeled amino acids. It was found that methionine from the liver proteins was one of the principal tissue source of the sulfur amino acids for the wound proteins. To some extent, the muscle proteins contributed to this requirement for the sulfur amino acids. It was also noted that a large part of the liver methionine was transformed into cystine for deposition in the regenerating wound protein. These experiments also produced evidence to show that the rate of metabolism of methionine and cystine was increased after wounding.

Of particular importance were the results which indicated that the tensile strength of the wound was closely correlated with the deposition of cystine in the regenerating wound proteins. The collagluous fibers in regenerated wound tissue give it a relatively high tensile strength. This type of protein contains appreciable amounts of the sulfur amino acids. Thus from the empirical and the theoretical view point, this correlation is sound. A new technique for determining the rate of healing of wounds is now being developed, based on the deposition of cystine in regenerating wound tissue.

The effect of various hormones on the healing of wounds was also studied. It was observed that estradiol, testosterone and pituitary thyrotropic hormone

inhibited the rate of healing. At high levels of administration, growth hormone also inhibited healing. However, at lower levels, there appeared to be an increased rate of healing. This aspect of the problem on the healing of wound is only preliminary. It is planned to pursue this work further.

Almost all of the important data supporting the above conclusions have been published or have been accepted for publication and will appear in print shortly. These publications are appended to this report. Included in the appendix are the following papers:-

"Metabolism of protein nitrogen during the healing of experimental wounds": Fed. Proc., 10, 270 (1950)

"Relation of protein nutrition to the healing of experimental wounds": Proc. Soc. Exp. Biol. Med., 77, 302 (1951)

"Effect of cystine and methionine on the healing of experimental wounds": Proc. Soc. Exp. Biol. Med., 80, 623 (1952)

"Inhibition of urea formation by pentobarbital": Arch. Biochem. Biophys., 32, 226 (1951)

"Distribution of methionine-S^{35} during the healing of experimental wounds": Fed. Proc., 12, 291 (1953)

"Utilization of sulfur amino acids during the healing of experimental wounds": Proc. Soc. Exp. Biol. Med., 83, 329 (1953)

"Influence of experimental wounds on the metabolism of sulfur": Fed. Proc., 13, 322 (1954)

"Influence of growth hormone on the healing of experimental wounds": Fed. Proc. 13, 325 (1954)

"Incorporation of sulfur amino acids into the proteins of regenerating wound tissue": J. Biol. Chem.: in press.

"Excretion of sulfur during the healing of experimental wounds": Proc. Soc. Exp. Biol. Med.: in press.

The chief investigator of this project is very much indebted to his assistants, T. H. McCarthy, H. J. Fromm, E. Bremanis and G. J. Neumann, who worked so arduously to help obtain the results which have been described. Without their devoted efforts this project could not have progressed as far as it has.

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Relation of Protein Nutrition to the Healing of Experimental Wounds.* (18759)

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A number of reports have indicated that the level of protein intake has a marked effect on the rate of healing of wounds(1-8). There is, however, little work relating protein metabolism or the effect of amino acids to the healing of wounds(4-6,9,10). It has been shown that methionine will increase the rate of healing of experimental wounds(4), whereas tryptophane, valine and lysine have no significant effect on the rate of healing(10). Since greater nitrogen retention will occur when extra methionine is fed(11,12), it seems probable that the methionine, rather than other essential amino acids, is the limiting factor in the healing process. In fact, injured dogs will retain methionine sulfur and incorporate it into protein, even when a negative nitrogen balance exists(13). Beyond the fact that a negative nitrogen balance appears shortly after wounding(14,15), little seems to be known about protein metabolism during the healing of wounds.

Further study may help elucidate the role of protein metabolism in the healing process. The present paper attempts to relate nitrogen and sulfur metabolism to the rate of healing of experimental wounds.

* This work was done under contract with the U. S. Navy, Office of Naval Research.

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2. Harvey, S. C., and Howes, E. L., *Ann. Surg.*, 1930, v91, 641.

3. Varco, R. L., *Surg. Gyn. Obstet.*, 1947, v84, 611.

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6. Thompson, W. D., Ravdin, I. S., and Frank, I. L., *Arch. Surg.*, 1938, v36, 500.

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12. Brush, M., Willman, W., and Swanson, P. P., *J. Nutr.*, 1947, v33, 389; Allison, J. B., Anderson, J. A., and Seeley, R. D., *J. Nutr.*, 1947, v33, 301.

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14. Werner, S. C., *Ann. Surg.*, 1947, v126, 169.

15. Koop, C. E., Drew, J. H., Riegel, C., and Rhoads, J. E., *Ann. Surg.*, 1946, v124, 1165.

TABLE I. Composition of Diets.*

Group #	% casein	% gelatin	% supplemental methionine	g N/100 g diet
I	20	0	0	2.56
II	6	0	0	.76
III	6	11.3	0	2.55
IV	6	11.3	.147	2.57

* Sucrose content of diets was adjusted so that all diets provided same number of calories/100 g diet.

Experimental. In these experiments, four groups of 24 female albino rats were used. The initial average weight of the animals in each group ranged from 170 to 186 g. The animals in Group I were fed the following basal diet: sucrose, 63 g; casein, 20 g; lard, 10 g; corn oil, 2 g; salt mixture,† 5 g; thiamine HCl, 1 mg; riboflavin, 1 mg; pyridoxine HCl, 1 mg; calcium pantothenate, 4 mg; nicotinic acid amide, 15 mg; inositol, 5 mg; 2-methyl naphthoquinone, 0.5 mg; choline chloride, 25 mg; vitamin A,‡ 1500 IU; vitamin D,‡ 210 IU. The other groups of animals were fed diets, modified from the basal, as indicated in Table I. All the diets were isocaloric and contained the same amount of vitamin supplement as in the basal diet. Each animal was presented with 15 g of diet per day. The residual uneaten food was weighed to determine the daily food consumption. All animals were given distilled water, *ad libitum*. The animals were first accustomed to the synthetic diets for 4 days. Then they were wounded under nembutal and ether anesthesia. After clipping the hair over the area of the shoulder blades, a skin incision, 2 cm in length, was made with a sharp scalpel in the mid-dorsal region. The edges of the wound were opposed and held in place with Michel wound-clips (11 mm), which were left in place during the experiment. At approximately weekly intervals, 8 rats from each group were killed and the tensile strength of a 0.5 cm section of the healing wound was determined. The method of measuring the tensile strength of such wounds has been previously described(1).

The rats were weighed and 24-hour urine samples were collected regularly during the course of the experiment. The urine samples were stored under toluene at 5°C until the analyses were made. The urine was analyzed for total nitrogen (microkjeldahl), urea nitrogen(16), amino acid nitrogen(17), and total sulfur(18).

Results and discussion. Previous papers have indicated that a negative nitrogen balance appears directly after animals have been subjected to trauma(9,13-15). A sharp decrease in the amount of nitrogen retained, shortly after the animals were wounded, was observed in these experiments, although not all the animals went into negative nitrogen balance. The sulfur balance remained positive and essentially constant throughout the experiment, regardless of the changes in the nitrogen balance. By the tenth day after wounding, the daily nitrogen retention had returned to approximately the level observed before the wound was made. This is illustrated in Table II.

A marked decrease in urinary urea output (from 109 mg per day to 81 mg per day) with a concomitant increase in the amino acid nitrogen output (from 6.1 mg per day to 26.6 mg per day) was observed shortly after the

TABLE II. Nitrogen and Sulfur Retention Before and After Wounding.

Group #	Avg retention before wounding*		Retention on 2nd day after wounding*		Retention on 10th day after wounding†	
	N, mg	S, mg	N, mg	S, mg	N, mg	S, mg
I	92	11.3	44	11.2	85	10.1
II	9	2.8	−11	1.5	5	2.3
III	84	6	14	3.8	39	6.6
IV	52	9.2	15	11.1	42	13.4

* Computed from analyses obtained from 24 animals per group.

† Computed from analyses obtained from 16 animals per group.

† Hubbel, R. B., Mendel, L. B., and Wakeman, J. J., *J. Nutr.*, 1937, v14, 287.

‡ From Oleum percomorphum

16. Van Slyke, D. D., and Cullen, G. E., *J. Biol Chem.*, 1916, v24, 117.

17. Northrup, J. H., *J. Gen. Physiol.*, 1926, v9, 767.

18. Fiske, C. J., *J. Biol. Chem.*, 1921, v47, 501

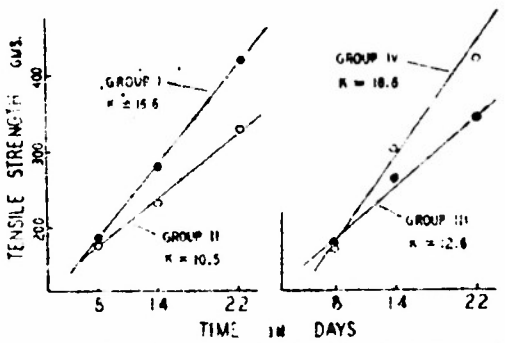

FIG. 1. Tensile strength plotted as function of time in days after wounding. The slope (K) is an index of the rate of healing.

wounds were made.§ The urea nitrogen and amino acid nitrogen returned to approximately the normal, prewounding levels within 5 days. This effect was found to be due to the anesthetic used and not to the wound.

In Fig. 1 is shown the rate of healing of the experimental wounds in terms of tensile strength and time. This can be expressed as, $T = K\,t$, where T is the tensile strength in grams per 1.5 cm section of healing epithelium, t, the time in days after wounding, and K, the resulting slope. The slope, K, can be considered to be a measure of the rate of healing of the wounds. It will be seen that the rate of healing of the wounds in the animals fed the 6 percent casein diet (Group II; K = 10.5) is markedly less than that of the animals on the 20% casein diet (Group I; K = 16.6). This is in agreement with previous work which has indicated that wounds in animals on a high protein diet heal more rapidly than those in animals on a low protein diet(1-4). From this, it would be expected that the rate of healing of the wounds in animals in Group III should be the same as those in Group I, since the protein nitrogen intake in both groups was the same. Instead, the slope (K = 12.6) indicates that the rate of healing is very much less than that found in Group I. This difference in healing rate is obviously due to the very low biological value of the gelatin, supplemented to the diet of the animals in Group III to make it contain the same amount of protein nitrogen as is found in the diet of Group I.

Although the proteins ingested, and the protein nitrogen intake, is essentially the same in the animals comprising Groups III and IV, the rate of healing of Group IV (K = 18.8) is very much greater than that for the animals in Group III. This difference must be attributed to the methionine added to the diet of the rats in Group IV. It can then be concluded that the methionine increases the efficiency of utilization of dietary protein or tissue protein, or both. Since the methionine fed is probably available to the tissues for protein synthesis as cysteine and cystine, as well as methionine(19,20), all the sources of dietary sulfur might be considered together as "protein sulfur."

There appears to be no correlation between the protein nitrogen intake, or retention, and the tensile strength or rate of healing, when all 4 groups of animals are considered. The data in Table III, however, indicate a correlation between the amount of "protein sulfur" retained during the course of the experiment and the rate of healing of the wounds. It seems evident from these results that previous reports(1-8) on the effect of the level of protein in the diet on the rate of healing were indicative of the effect of the level of "protein sulfur" intake (and indirectly, the sulfur retention), rather than the effect of the protein itself. This is apparent from the fact that previous reports dealt with the effect of diets containing levels of the same protein; in which case, the sulfur content of the diets varied directly with the amount of protein in the diet.

The data in Tables II and III tend to indicate a larger proportion of sulfur to nitrogen is required during healing than for normal tissue synthesis. This is supported by the following: (a) after wounding, a positive sulfur balance is observed even when there is a negative nitrogen balance. This may mean that there is a sacrifice of tissue protein nitrogen so that the ratio of sulfur to nitrogen

§ Data represent average urinary output of all animals in these experiments.

19. Binkley, F., *J. Biol. Chem.*, 1944, v155, 39.

20. DuVigneaud, V. J., Kilmer, G. W., Rachele, J. R., and Cohn, M., *J. Biol. Chem.*, 1944, v155, 645.

TABLE III. Comparison of Avg Daily Intake and Retention of Dietary Nitrogen and Sulfur and Rate of Healing.*

Group #	Nitrogen		Sulfur		Rate of healing index†
	Avg daily intake, mg	Avg daily balance, mg	Avg daily intake, mg	Avg daily balance, mg	
I	266	78	13.2	11.8	16.6
II	78	10	3.6	2.6	10.5
III	269	47	8.2	6.4	12.6
IV	251	56	23.1	14	18.8

* These values indicate daily avg for 21 days after wounding.
† See Fig. 1.

available is increased; (b) increasing the ratio of sulfur to nitrogen by dietary means (by the use of methionine) increases the rate of healing; (c) healing wound tissue contains larger amounts of sulfur than the same tissue when it has not been wounded(21,22).

Thus it seems probable that the rate of healing may be related to, among other factors, the amount of "protein sulfur" retained in excess over that utilized to form normal tissue protein with the retained nitrogen. Since the ratio of nitrogen to sulfur in most tissues is about 16:1, then the healing should be influenced by the amount of retained "protein sulfur," less 1/16 the quantity of retained nitrogen. This might be called the "excess sulfur" which is available for the greater demands of the healing wound tissue. The "excess sulfur" is 156, 44, 75 and 214 mg for the animals in Groups I to IV, respectively. Thus, there appears to be some correlation between the rate of healing and the "excess sulfur" available for the wound.

Summary. Wounded rats were kept on diets containing varying amounts of different proteins and "protein sulfur". There appears to be no relation between the amount of protein nitrogen fed or retained by the rats and the rate of healing. However, the "protein sulfur" retention appears to be correlated with the rate of healing. It is suggested that the amount of retained sulfur in excess over that utilized for normal tissue protein synthesis is an important factor in determining the rate of healing of experimental wounds.

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METABOLISM OF PROTEIN NITROGEN DURING THE HEALING OF EXPERIMENTAL WOUNDS

M. B. Williamson, T. McCarthy and H. Fromm

It has been shown that wounds heal more rapidly when a high protein diet is supplied than when a low protein diet is given. When isocaloric diets containing 0.49% and 1.94% protein nitrogen (as casein) were fed to groups of 24 rats, the rate of healing of experimental wounds, as measured by increase in tensile strength(Science, 105: 396, 1947), is greater in the animals fed the 1.94% protein N diet than in those on the 0.49% protein N diet. However, the increase in tnesile strength per total nitrogen available for healing was much greater on the low protein diet, even when there was a negative nitrogen balance, than on the high protein diet. This seems to indicate that more of the plasma and tissue protein nitrogen is being utilized for protein synthesis in the wound area on the low N diet than on the high N diet. Supplementing the low protein diet with gelatine so that the nitrogen content of the diet was the same as that of the high protein diet had little effect on the rate of healing of the wounds, but markedly increased the excretion of nitrogen. Supplementing the low protein N diet with gelatin and methionine so that it contained as much nitrogen and methionine as the high protein diet increased the rate of healing, and decreased the nitrogen

excretion to approximately the level found in the rats fed the high protein N diet. The rats on the low protein N diet retained a higher percentage of the dietary sulfur than did the animals on any of the other diets.

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Distribution of Mehtionine-S^{35} During the Healing of Experimental Wounds

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The rate of healing of experimental wound has been shown to increase upon the addition of either cystine or methionine to the diet. The effect of these sulfur amino acids on the regeneration of wound tissue was studied by investigating the distribution of methionine-S^{35} between the methionine and cystine sulfur of wound tissue, normal skin, liver and muscle. Rats fed either a zero protein diet, or a zero protein diet supplemented with methionine (35 Cal/day), were injected with methionine-S^{35} in tracer amounts within 48 hours after a standard wound was made. At 3 day intervals, part of each group of rats was sacrficed and tissue samples obtained. The total sulfur, nitrogen, cystine and methionine, as well as the distribution of S^{35}, were determined. Within 5 days, the regenerating wound tissue was found to contain twice as much S^{35} as the normal unwounded skin tissue. The amount of total S^{35} in the regenerating tissue continued to increase with time. There appeared to be a direct correlation between the rate of increase of S^{35} in the wound and the rate of healing. The total S^{35} remained constant in the unwounded skin tissue, while that in the liver decreased with time. Although the total S^{35} in the muscle decreased with time on a zero protein diet, it remained constant when methionine was fed. In the

wound tissue, most of the S^{35} activity was found associated with with the cystine. The ratio of cystine to methionine sulfur was higher in the wound tissue than in the normal unwounded tissue, incidating that a different type of protein was being produced during regeneration.

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Utilization of Sulfur Amino Acids During Healing of Experimental Wounds.* (20350)

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The regeneration of tissue during the healing of wounds has been shown to be markedly accelerated by the addition of methionine to the diet(1-7). More recently, the rate of healing of experimental wounds was shown to be increased to the same extent when equivalent amounts of either cystine or methionine were administered(1). Since the conversion of methionine to cystine is irreversible *in vivo* (9,10), this work may be taken to indicate that cystine is the limiting amino acid in the healing process. The possibility still existed that the sulfur amino acids may not be required *per se*, but rather might serve as a source of sulfur for other compounds required

* This work was done under contract with the U. S. Navy, Office of Naval Research.

during healing, as well as their more usual function in protein synthesis.

It has been shown also that there is a small but definite retention of sulfur during healing, even when a negative nitrogen balance is in evidence(1,2,8). Further, the retention of sulfur becomes more significant as the rate of healing increases. Thus, there appears to be a correlation between the sulfur retention and the *healing index*. The *healing index* is a measurable numerical evaluation of a function which is directly proportional to the rate of healing(1,2). The metabolic fate of sulfur and sulfur containing amino acids during the healing of experimental wounds should give an insight into the disposition of the retained sulfur. The utilization of the cystine by the regenerating wound tissue and its relation to the function measured by the *healing index* are considered in this report.

Experimental. The experiments to be described were carried out on two groups of female albino rats (18 animals per group), weighing 170 ± 20 g. The animals were maintained on a basal 0% protein diet for 5 days prior to wounding. After wounding, one group of rats was continued on the 0% protein diet supplemented with 60 mg of DL-alanine per 100 g of diet, while the second group received the 0% protein diet supplemented with 100 mg of DL-methionine per 100 g of diet. The basal diet consisted of 83 g sucrose, 10 g lard, 2 g corn oil, 5 g salt mixture(15), 1 mg thiamine HCl, 1 mg riboflavin, 1 mg pyridoxine HCl, 4 mg calcium pantothenate, 10 mg nicotinic acid amide, 0.5 mg 2-methyl-naphthoquinone, 1550 IU vit. A,† and 210 IU vit. D.† The animals were provided with 7 g of diet per day, which was completely consumed before the next daily feeding period. Distilled water was permitted *ad libitum*. After removing the hair, the wounds were made on the back of the neck of the rats by tracing the outline of a coin (3 cm diameter), and cutting on the marked tracing. The circle of skin was cut down to the layer of the *fascia* and removed with any adhering connective tissue, according to the method described by Paul *et al.*(16). Two days after wounding each rat was injected subcutaneously with 0.85 mg of S^{35}-labeled L-methionine, representing 5.9×10^6 counts per minute. One-third of the animals in each group were sacrificed on the 5th, 8th and 12th day after wounding. Samples of regenerating wound tissue, normal unwounded skin, liver and muscle were taken from each rat and hydrolyzed in 6 N HCl. Great care was taken to insure that no normal tissue was included in the sample with the regenerating wound tissue. Normal unwounded skin tissue was obtained from the area near the site of the wound. This tissue was completely freed of hair before analysis. The above tissue samples were analyzed for total sulfur(11), total nitrogen (microkjeldahl), and cysteine + cystine (designated as *cystine*)(12,13). The relatively small amounts of sample available made it necessary to calculate the methionine present in the tissues by difference. Although the methionine-sulfur fractions probably included sulfur from compounds other than methionine, by far the largest part of these fractions of the sulfur comes from the methionine. Besides determining the total S^{35} in the tissue samples, aliquots were partitioned into cysteine + cystine and non-cysteine + cystine fraction by the method of Zittle and O'Dell(14), and the S^{35} determined in the fractions. The latter fraction was designated as the methionine-S^{35} fraction.

Results and discussion. In Table I is shown the S^{35} content of the various tissues which were analyzed in terms of counts per minute per gram of tissue. It should be noted that there is a very marked increase in the S^{35} content of the regenerating wound tissue with time. This increase reflects the accretion of

† From *oleum percomorphum*.

TABLE I. Effect of Dietary Methionine on Distribution of Methionine-S^{35} in Wounded Rats.

Days after wounding	Counts/min./g of tissue			
	Wound tissue	Skin	Liver	Muscle
	0% protein diet			
5	6065	2227	7986	1660
8	7222	1856	5154	1324
12	6371	1630	4220	924
	0% protein diet + methionine			
5	6272	2705	7175	1252
8	9417	2267	5030	1157
12	11565	1967	3740	865

TABLE II. Sulfur and Nitrogen Content/g of Wounded Rat Tissue.

Days after wounding	Wound tissue S	Wound tissue N	Skin S	Skin N	Liver S	Liver N	Muscle S	Muscle N
			mg					
			0% protein diet					
5	1.9	20.2	2.0	39.9	3.0	34.0	2.8	34.4
8	2.3	22.0	1.7	40.8	2.5	31.5	2.7	33.5
12	2.9	27.8	1.6	42.4	2.3	28.7	2.3	35.6
			0% protein diet + methionine					
5	2.0	22.4	2.1	39.2	2.9	30.5	2.7	34.2
8	2.8	26.3	1.6	42.1	2.5	27.1	2.5	36.1
12	3.6	29.8	1.6	40.2	2.3	26.7	2.4	35.6

TABLE III. Distribution of S^{35} from Methionine between Cystine and Methionine Fractions of Tissue in Wounded Rats.

Days after wounding	Wound tissue Cystine*	Wound tissue Methionine	Skin tissue Cystine*	Skin tissue Methionine
	Counts/min./g tissue			
	0% protein diet			
5	3536	2417	1465	910
8	4580	3205	1370	730
12	5240	4026	1171	452
	0% protein diet + methionine			
5	3007	2027	1427	1377
8	5177	3600	1085	1225
12	6930	4072	940	1003

* The "cystine" fraction includes both cysteine and cystine.

new cells and protein in the wound area. Due to metabolic turnover, the S^{35} content of the other tissues decreased with time. Although some small part of the methionine-S^{35} may have been directly utilized, there can be little doubt that the bulk of the wound tissue S^{35} arose from the degradation products of other tissue proteins.

The total sulfur and nitrogen content of these tissues is tabulated in Table II. The general trend of the total sulfur levels in the tissues parallels that observed for the S^{35}. However, the changes observed are not as marked as in the case of the radio-sulfur. This may account for the previous difficulty in obtaining significant differences in the sulfur content of wound tissues which had been healing for different lengths of time. Particular note should be made of the fact that the sulfur content of the regenerating wound tissues is very much higher than that found in the other tissues analyzed, based on the nitrogen content of the tissues.

The only significant change in the nitrogen content of the various tissues occurred in the case of the wound tissue, although the small decreases observed in the liver may be reflecting the negative nitrogen balance which existed in these animals. The relatively low nitrogen concentrations appearing in the healing wound tissue samples are consonant with previous work which had shown that this type of tissue has a very high water content(16). From the data in Table II, the water content of the wound tissue appears to diminish gradually with the progression of the healing process.

In Table III, the results of the partition of the S^{35} into *cystine* and methionine-S^{35} fractions is shown. It is of special importance to note that so large a proportion of the injected methionine-S^{35} is converted to cysteine- or cystine-S^{35}. In agreement with the data shown in Table I, the sum of the two S^{35} fractions increases with time. However, the concentration of the *cystine*-S^{35} is much greater than is that of the methionine-S^{35}. These results would appear to support the previous report(1) showing that although methionine is required during the healing process, there appears to be greater utilization of cystine.

Table IV shows the results of the analysis of the tissues for *cystine* and methionine. The regenerating wound tissues accumulate *cystine* quite rapidly while the concentration of methionine increases at a more leisurely rate. In the liver and muscle, there was a marked decrease in the methionine concentration while the *cystine* remained essentially unchanged throughout the experiment. This might be taken to indicate that the liver and muscle

TABLE IV. Cystine* and Methionine Content/g Tissue in Wounded Rats.

Days after wounding	Wound tissue		Skin		Liver		Muscle	
	Cys	Meth	Cys	Meth	Cys	Meth	Cys	Meth
				mg				
				0% protein diet				
5	3.6	4.0	3.0	5.2	4.2	7.9	4.1	7.5
8	5.3	4.2	2.9	5.3	5.0	6.9	4.3	7.4
12	7.3	5.4	2.9	5.4	4.7	4.4	3.9	6.1
				0% protein diet + methionine				
5	4.0	4.1	3.0	4.9	4.5	8.3	4.1	8.3
8	6.7	4.7	2.7	4.9	4.6	6.6	4.1	6.5
12	9.5	5.9	2.8	4.5	5.1	4.7	4.0	6.3

* The "cystine" fraction includes both cysteine and cystine.

methionine sulfur are the principal sources for the wound tissue sulfur. It would also follow that a large portion of the metabolized liver and muscle methionine is converted to cysteine or cystine, which may be utilized by the regenerating wound tissue. This is supported by the fact that, in the animals fed the 0% protein diet, the ratio of *cystine* to methionine excreted was found to be approximately 5:1.

Previously reported analyses, as well as those shown here, indicate that there is usually appreciably more methionine found in most tissues, than there is *cystine*. This, however, is not the case in the regenerating wound tissue. In the wound tissue, the concentration of *cystine* becomes increasingly greater than the level of methionine during the course of healing. This fact might be interpreted to mean that there are two types of proteins being synthesized in the wound area. The first type might be considered to be synthesized relatively slowly and to contain a higher proportion of methionine than *cystine*. The characteristics of this type of protein are probably similar to skin proteins in general. The second type, although not synthesized until at least some of the first type of protein has already been laid down in the wound area, is synthesized more rapidly and contains a higher proportion of *cystine* than methionine. Fractionation studies of the proteins in regenerating wound tissue at various stages of development, and in normal skin tissue, are now under way to test this hypothesis.

The rates of healing of wounds in rats fed a 0% protein diet and a 0% protein diet supplemented with methionine were determined as previously described (1,2). The results are shown in Fig. 1 to be essentially straight lines of different slopes (*healing indices*). When the *cystine* per gram of wound tissue is plotted against time, straight lines also result (Fig. 1). These lines may be represented by the following equations:

$$C = k_1 t + a$$
$$T = k_2 t + b$$

where C is the *cystine* concentration in the wound tissues, T, the tensile strength, t, the time in days after wounding, and the other symbols represent constants. It would then follow that: $C = k_3 T + c$. If this equation is valid, a straight line should result from a plot of tensile strength against the *cystine* content of the wound tissue. That this is actually the case is shown in Fig. 2. It may then be concluded that the rate of deposition of *cystine* in the wound tissue is proportional to the rate of increase in tensile strength of the wound (healing index). Since the rate of healing is proportional to the *healing index*, it can now be measured by two separate functions, the tensile strength of the wound, and the cystine content of the wound area.

It might be assumed that the measurement of the tensile strength of wound tissue, requires, among other factors, the rupture of bonds between relatively large molecules which are not in solution, but are arranged in some sort of colloid meshwork. It would then seem probable that the *cystine* in the wound tissue is preponderantly, if not completely, associated with protein molecules.

Summary. Wounded rats, injected with L-methionine-S^{35}, accumulate the radio-sulfur in the wound tissue, even when the S^{35} content of the other tissues is decreasing. The largest

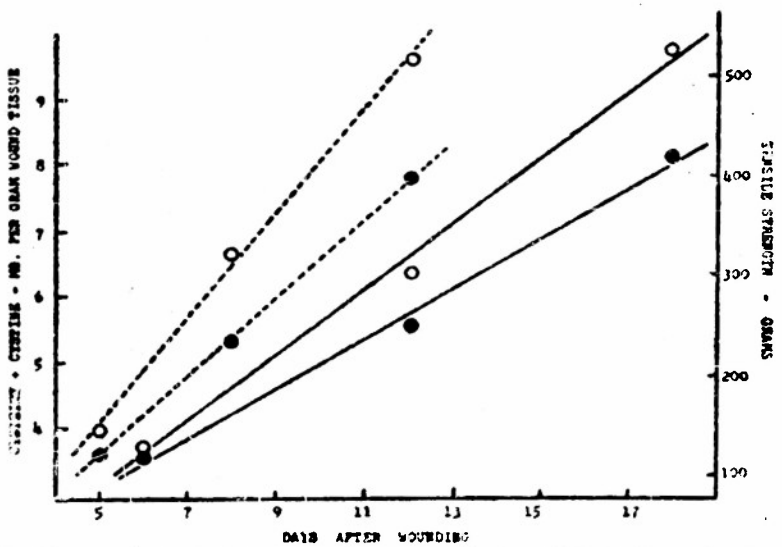

FIG. 1. Tensile strength and cystoine + cystine concentration of healing wounds in rats on a non-protein diet plotted against time. Solid circles, diet supplemented with alanine; open circles, diet supplemented with methionine. Solid lines, tensile strength data; broken lines, cysteine + cystine data. Significance between the mean values of tensile strength is "p" <0.02. Significance between the mean values of the cysteine + cystine is "p" <0.05.

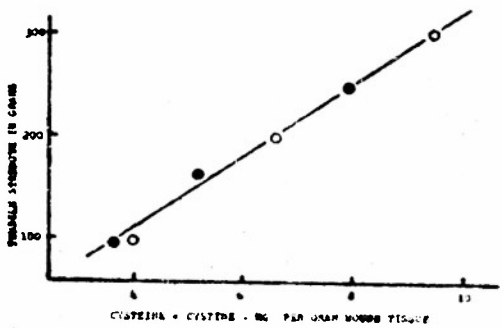

FIG. 2. Tensile strength of healing wound in rats on a non-protein diet plotted against the cysteine + cystine concentration of the regenerating wound tissue. Solid circles, diet supplemented with alanine; open circles, diet supplemented with methionine.

part of the S^{35} in the wound tissue appears as cystine-S^{35}. The rate of deposition of cystine in the regenerating wound tissue is greater than that of methionine. The rate of healing (as measured by the *healing index*) is a function of the cystine content of the wound tissue.

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Effect of Cystine and Methionine on Healing of Experimental Wounds.* (19712)

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In previous reports(1,2), the more rapid rate of healing of experimental wounds in animals fed a high protein diet as compared to those on a lower protein diet was shown to be due to the greater intake and retention of protein sulfur. It was also shown that methionine could serve as the source of protein sulfur. This explained earlier work which had indicated that methionine increased the rate of healing(3-5). Other essential amino acids (6,7) were found to have no effect on the *healing index*, a numerical measure of a function which is proportional to the rate of healing.

Methionine may have 2 possible roles, involving its sulfur atom, which might affect the *healing index*. First, it might be required directly in the healing processes for such reactions as protein synthesis. Secondly, methionine might serve as a precursor for some other sulfur-containing compound required during healing. Although these alternatives need not necessarily be mutually exclusive, it is not unreasonable to presume that one function will be more important than the other.

In the present paper, it is shown that the *healing index* can be affected by cystine to the same extent as by an equivalent amount of methionine, on the basis of sulfur.

Experimental. The experiments to be described were carried out in a similar manner to those previously reported(1,2). In each experiment, 3 groups of 24 female albino rats (200 ± 20 g) were maintained on a basal diet for 5 days prior to wounding. The basal diet consisted of 6 g casein, 10 g lard, 2 g corn oil, 5 g salt mixture(8), 77 g sucrose, 1500 I.U. vit. A,† 210 I.U. vit. D,† 1 mg thiamine HCl, 1 mg riboflavin, 1 mg pyridoxine HCl, 15 mg nicotinic acid amide, 4 mg calcium pantothenate, 0.5 mg 2-methyl naphthoquinone, 5 mg inositol, and 25 mg choline chloride. This diet would permit only a small amount of protein accretion in normal unwounded animals (as measured by nitrogen excretion and increase in body weight). On the day of wounding, the animals were transferred to the experimental diets. All the animals were given the same weighed amount of diet daily, in quantities which would be completely consumed. Distilled water was permitted *ad libitum*.

Standard experimental wounds were made on the back of the neck of the rats as previously described(1). At approximately weekly intervals, 1/3 of the animals in each group were sacrificed and the tensile strength of a number of 0.5 cm sections of the healing wound were measured(1,9). The relationship of tensile strength to time results in a curve which may be considered to be essentially a straight line. This line can be represented by the equation $T = kt + C$, where T is the tensile strength in grams, and t, the time in days. C is a constant. The slope of this line (K) is the *healing index*, and may be computed from the equation:

$$K = \frac{T_2 - T_1}{t_2 - t_1}$$

where T_1 is the tensile strength at time t_1, and T_2 is the tensile strength at time t_2. K

* This work was done under contract with the U. S. Navy, Office of Naval Research.

† From oleum percomorphum.

TABLE I. Effect of Experimental Wounds on the Sulfur and Nitrogen Balances. Exp. I.

Group No.	Casein in diet, %	Amino acid supplement	Nitrogen per 100 g diet, mg	Sulfur per 100 g diet, mg	Nitrogen — Avg daily intake, mg	Nitrogen — Avg daily retention, mg	Sulfur — Avg daily intake, mg	Sulfur — Avg daily retention, mg	Excess sulfur,* mg	*Healing index*
I	6	Alanine	910	48	64.8	[illegible]	17.7	1.53	1.07	40
II	6	Methionine	910	248	64.8	22.1	17.7	4.15	2.68	50
III	6	Cystine	910	248	64.8	22.7	17.7	4.22	2.64	51

has the dimension of a rate term which describes a function of the rate of healing.

Urine samples were collected daily before and after the time of wounding. The urine was stored under toluene at 5°C until analyses were run. The urine was analyzed for total nitrogen (microkjeldahl) and sulfur (10,11).

Results and discussion. The nitrogen and sulfur sources of the diet fed the wounded animals used in Exp. 1 are described in Table I. The *healing indices* for these animals were calculated from the tensile strength data plotted in Fig. 1. It can be seen that the rates of healing in the animals receiving the methionine and cystine supplements (K = 50 and 51) are significantly greater than that of the control group (K = 40). It should be noted particularly that equivalent amounts of cystine and methionine (on the basis of sulfur) have the same effect on the *healing index*. A repetition of this experiment gave identical results.

A correlation between the sulfur retention and the *healing index* was observed here, as in previously reported work (1,2). It would be expected that the retention of sulfur should be proportional to the retention of nitrogen, in a ratio similar to that found in the animal. In the rat, the nitrogen:sulfur ratio is approximately 15:1. After wounding, there appears to be a greater retention of sulfur than might be expected from the amount of nitrogen which is retained. This "excess sulfur" also appears to be correlated with the *healing index*. The data supporting this correlation are shown in Table I.

Whether the results noted above were due to the direct action of the cystine supplement, or to the effect of the cystine in sparing the methionine available from the casein in the diet, remained to be determined. Therefore, an experiment similar to the previous one was carried out, except that the casein was omitted from the diet fed the wounded animals. The control group of animals received a diet containing no sulfur amino acids and 44 mg of amino acid nitrogen per 100 g diet, in the form of alanine. The cystine and methionine supplemented diets contained 100 mg of amino acid sulfur and 44 mg of nitrogen per 100 g of diet. The curves of tensile strength against time obtained in this experiment are plotted in Fig. 2.

Here again, the effect of cystine and methionine on the *healing index* can be seen to be essentially the same (K = 36 and 34) and significantly greater than that found in the

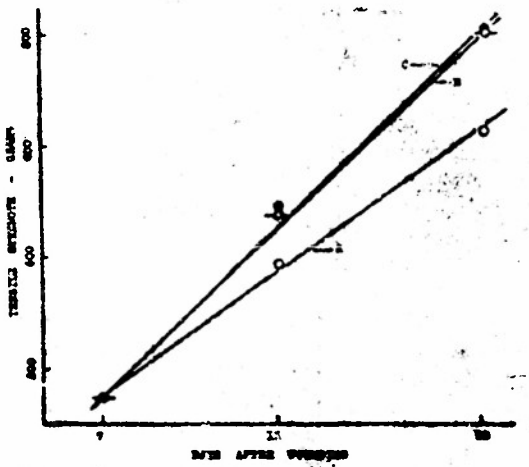

FIG. 1. Tensile strength of healing wounds in rats on a 6% casein diet plotted against time. Curve A (Group I), alanine supplement, *healing index* (K) = 40; Curve B (Group II), methionine supplement, (K) = 50; Curve C (Group III), cystine supplement, (K) = 51. The significance between mean values of tensile strength for Groups I and II is "p" = <.01.

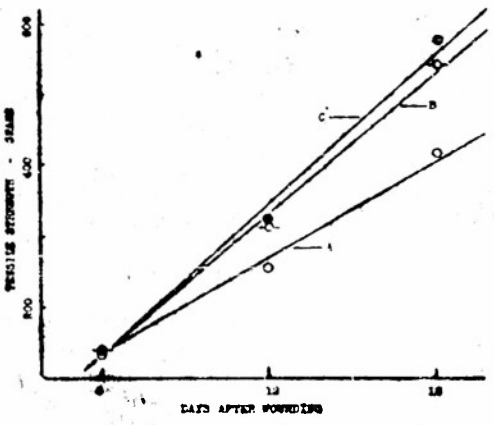

FIG. 2. Tensile strength of healing wounds in rats on a non-protein diet plotted against time. Curve A (Group I), alanine supplement, *healing index* (K) = 24; Curve B (Group II), methionine supplement, K = 34; Curve C (Group III), cystine supplement, K = 36. Significance between mean values of tensile strength for Groups I and II is "p" . <.01

controls (K = 24). Since the conversion of methionine to cystine is irreversible *in vivo* (12,13), it must be concluded that the methionine in the diet is first converted to cystine before it becomes available for the healing processes. It then appears that cystine is the limiting factor affecting the *healing index*, and that methionine serves primarily as a source of cystine sulfur. Of course, cystine, as such, may not be required by the healing wound. It may be that cystine is itself merely a precursor of the sulfur containing substance utilized during healing. The correlations between the *healing indices*, the sulfur balances and the "excess sulfur" values for this experiment are shown in Table II.

Further work has indicated that methionine, *per se*, is required to some extent during wound healing, over and above that which may be converted to cystine. When rats were fed a 5% casein diet, supplemented with 100 mg of ethionine sulfur and 100 mg of either cystine or methionine sulfur per 100 g diet, a lower *healing index* was observed than in the control animals, who received no sulfur amino acid supplement. A comparison of the tensile strength data for this experiment is shown in Fig. 3. These data may be interpreted to mean that the ethionine is interfering with the utilization of both the cystine and the methionine.

Ethionine is known to block the conversion of methionine to cystine as well as the incorporation of methionine into protein (14,15). The latter effect results in a decreased rate of protein synthesis. In Group II (methionine supplement), the low *healing index* may be considered to be due to the lack of cystine resulting from the interference with methionine conversion. However, in spite of the relatively large amounts of cystine available to the animals in Group III, a low *healing index* was still observed. In this case, it seems probable that the ethionine interfered with the utilization of methionine for purposes other than cystine formation, so that the cystine requirement was no longer the limiting factor in the healing process. It must then be concluded that methionine is also required for wound healing. It is not unlikely that the methionine requirement during wound healing is needed primarily for protein synthesis, whereas, the cystine required may be used, to some extent, for reactions other than protein synthesis.

In the experiment where the diet fed to the wounded rats contained no protein, the methionine required for healing by the control animals and by those receiving the cystine supplement must have originated in the tissue protein. It would then be reasonable to think that the methionine requirement must be

TABLE II. Effect of Experimental Wounds on Nitrogen and Sulfur Balances. Exp. II.

Group No.	Nitrogen		Sulfur			
	Avg daily intake, mg	Avg daily retention, mg	Avg daily intake, mg	Avg daily retention, mg	Avg "excess sulfur," mg	*Healing index*
I	3.1	−40.1	6	−2.06	.60	24
II	3.1	−35.9	7	1	1.59	34
III	3.1	−35.6	7	1.04	1.33	36

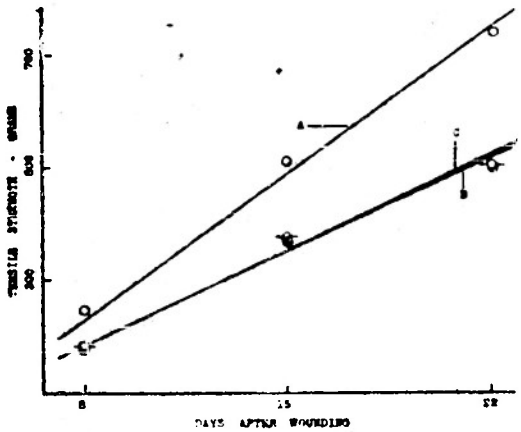

FIG. 3. Tensile strength of healing wounds in rats on a 5% casein diet plotted against time. Curve A (Group I), alanine supplement, *healing index (K)* = 31; Curve B (Group II), ethionine and methionine supplement, $K = 21$; Curve C (Group III), ethionine and cystine supplement, $K = 21$. Significance between mean values of tensile strength for Groups I and II is "p" = .01.

relatively small as compared to the cystine requirement.

Summary. The effect of methionine and cystine on the *healing index* of standard experimental wounds in rats was determined. Since both amino acids have the same effect, per equivalent of sulfur, it is concluded that methionine is converted to cystine before being used in the healing process. When the utilization of methionine is blocked by ethionine, cystine is ineffective, indicating that some methionine, *per se*, is required for the healing of wounds. There appears to be a correlation between the *healing index* and the retention of amino acid sulfur in excess of that expected on the basis of nitrogen retention.

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1064. Influence of experimental wounds on metabolism of sulfur. MARTIN B. WILLIAMSON AND HERBERT J. FROMM. *Dept. of Biochemistry, School of Medicine, Loyola Univ., Chicago, Ill.*

It has been shown that sulfur metabolism in wounded animals is altered from the normal. This problem was studied by investigating the nitrogen and sulfur balances in wounded and in normal rats (200 ± 20 gm) fed a 0% protein diet, supplemented with either cystine or methionine. At intervals after wounding, a number of the rats were sacrificed, in order to determine the sulfur amino acid content of the regenerating wound tissue, as well as various other tissues. It was found that the wounded animals excreted slightly more sulfur, but appreciably more nitrogen, than the unwounded rats. The sulfate ion excretion in the wounded rats was much greater than that observed in the normal animals. This was counterbalanced by a lower output of non-sulfate sulfur. These results may be taken to indicate an alteration in the path of sulfur metabolism. The tissue analyses showed that there was a gradual accretion of both cystine and methionine in the regenerating wound tissue. However, the rate of accretion of cystine was very much greater than that observed for the methionine, and appeared to be proportional to the rate of healing. This was observed in all the wounded animals, regardless of the diet fed. The cystine content of the other tissues remained essentially unchanged throughout the experiment. There was a marked decrease in the methionine content of the liver, while that of the other tissues remained constant or decreased only slightly, suggesting that the liver is one of the primary sources of methionine and cystine for the regenerating wound tissue.

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Inhibition of Urea Formation by Pentobarbital[1]

While investigating protein metabolism during the healing of experimental wounds, it was observed that a marked decrease in urea nitrogen and a simultaneous increase in amino acid nitrogen in the urine appeared within a short time after the wounds were made. At first, this reaction was attributed to the effect of the wound on protein metabolism. However, further study revealed that this effect could be produced by

TABLE I

Effect of Pentobarbital on the Excretion of Urea and Amino Acid N

No. of rats	Diet	Average prior to pentobarbital		After pentobarbital, 12–36 hr	
		Urea N	Amino acid N	Urea N	Amino acid N
		mg.	mg.	mg.	mg.
24	Casein, 20%	112	8.0	97	30.8
24	Casein, 6%	74	7.5	35	18.5
24	Casein, 6%; gelatin, 12%	140	4.7	110	31.2
24	Casein, 6%; gelatin, 12%; methionine, 0.04%	102	5.6	82	24.5
6	Stock diet	120	25.6	88	55.9

the administration of 15 mg./kg. body wt. of pentobarbital, which had been used as the anesthetic. This effect on the urea and amino acids excreted in the urine was obtained in all animals, regardless of the level or quality of the protein intake, and whether they had been wounded or not. In all cases, the urinary level of the urea and amino acid nitrogen returned to normal within 5 days after the administration of the pentobarbital.

The data shown in Table I were obtained from mature female rats (160–190 g.) which had been kept on the indicated diets for at least 4 days prior to the collection of the urine samples for analysis. The urine was collected for 24-hr. periods and kept under toluene at 5°C. until the analyses were made. The urea content of the urine was determined by a urease method (1), while the amino acid nitrogen was estimated by a modified formol titration (2).

The effect of the pentobarbital appears to be an inhibition of urea formation with a consequent increase in amino acid excretion. This effect may be due to an interference with one or more of the enzymes which deaminate amino acids, or which are involved in the conversion of these amino groups to urea in the liver. Experiments to study the effect of pentobarbital on the enzyme systems which are concerned with urea formation are contemplated.

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1965. **Influence of growth hormone on healing of experimental wounds.** MARTIN B. WILLIAMSON AND GEORGE J. NEUMANN. *Dept. of Biochemistry, School of Medicine, Loyola Univ., Chicago, Ill.*

Since the pituitary growth hormone has been shown to cause an increased retention of nitrogen, its effect on the healing index (rate of healing as measured by change in tensile strength of standard wounds with time) was studied. Female rats (200 ± 20 gm) were fed a 6% casein diet 1 wk. prior to wounding and thereafter to the end of the experiment. The wounding procedure has been previously described (*Proc. Soc. Exper. Biol. & Med.*, 80: 623, 1952). At about weekly intervals, ½ of the rats were sacrificed and the tensile strength of the wound determined. A significantly lower healing index was obtained when 1.0 mg/day of growth hormone was administered, than was found for the wounds in the control animals, which had been injected with isotonic saline. However, during the course of the experiment (20 days), the control rats lost 16 gm in weight, while those treated with growth hormone lost only 5 gm. When identical experiments were carried out using only 0.5 mg growth hormone/day, the healing index of the control animals was now lower than that of the growth hormone-treated rats. In this case, the control rats lost 14 gm in weight, while the hormone-treated rats lost 9 gm. These results would seem to indicate that large amounts of growth hormone mobilise protein metabolites primarily for use by the body tissue to the detriment of the healing wound tissue; smaller amounts of growth hormone permit greater diversion to the regenerating wound tissue.

THE INCORPORATION OF SULFUR AMINO ACIDS INTO THE PROTEINS OF REGENERATING WOUND TISSUE*

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Not only the administration of methionine, but that of equivalent amounts of cystine as well, has been shown to increase the rate of regeneration of wound tissue (1-6). Since the conversion of cystine to methionine does not occur to any appreciable extent in rats (7,8), it was considered that the deposition of protein in regenerating wound tissue is limited by the amount of cystine available for this process. Although regenerating wound tissue contains a relatively high concentration of cystine and methionine, as compared to normal unwounded tissue, the nitrogen content of both types of tissue are essentially the same (6, 9). These analyses indicate a fundamental difference between the proteins of wound tissue and the normal tissue which it is replacing. The incorporation of the sulfur amino acids in regenerating wound tissue, as well as the source of these amino acids will be considered in this report.

EXPERIMENTAL

In all of the experiments to be reported, female albino rats weighing 200 ± 20 grams were used. The animals were maintained on a basal diet for five days prior to wounding. This basal diet consisted of 83 g. sucrose, 10 g. lard, 2 g. corn oil, 5 g. salt mixture (11), 1 mg. thiamine HCl, 1 mg. riboflavin, 1 mg. pyridoxine HCl, 4 mg. calcium pantothenate, 5 mg. inositol, 5 mg. p-amino benzoic acid, 15 mg. nicotinic acid amide, 25 mg. choline chloride, 0.5 mg. 2-methyl naphthoquinone, 1500 IU vitamin A** and 210 IU vitamin D**. The animals were offered 8 g. of diet per day, which was completely consumed before the next daily feeding, so that the

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* This work was done under contract with the U. S. Navy, Office of Naval Research.

** From oleum percomorphum.

dietary intake of all the animals in each experiment was isocaloric. Distilled water was permitted ad libitum. After the acclimation period of five days, the basal diet was altered as will be indicated later.

On the sixth day, after the removal of the hair on the back of the neck and shoulders, the outline of a coin (4 cm. in diameter) was made with ink and the tissue excised down to the fascia, according to the procedure described by Paul, et al. (9). At intervals after wounding, the rats were killed and samples of regenerating wound tissue, normal skin tissue, muscle and liver were obtained. Special care was taken to insure that no normal tissue or hair was included with the samples of wound tissue.

The tissues collected were weighed wet and hydrolyzed immediately in 20% HCl - 50% HCOOH for 24 hours, so that minimal destruction of the sulfur containing amino acids resulted (10). Aliquots of the hydrolysate were then analyzed for nitrogen (microkjeldahl), cystine + cysteine (referred to as cystine) (12) and methionine (13). In the experiment in which tracer doses of S^{35} labeled DL-cystine were administered, the total S^{35} activity was determined after oxidation of an aliquot of the hydrolysate with a mixture of HNO_3 and $HClO_4$ (14). The resulting sulfate was precipitated as the barium salt and measured at "infinite thickness". The activity was measured using a Tracerlab Autoscalar and Geiger-Muller Tube having a window thickness of 1.4 mg per cm^2. The total S^{35} activity in these samples were assumed to be derived from cystine-S^{35}.

When tracer doses of DL-methionine, labeled with S^{35}, were given, it was assumed that some part of the methionine had been converted to cystine before deposition in the tissue proteins. The cystine was separated from the methionine in each sample by precipitation as the cuprous mercaptide (15). The S^{35} activity in these fractions was then determined as described above.

Exhaustive trituration and extraction of the regenerating wound tissue and the tissue removed to form the wound, with 0.1 N HCl, 0.1 N NaOH or dilute salt solutions gave rise to no sulfur containing compounds which were not precipitable with

trichloroacetic acid. It was then concluded that the sulfur amino acids in these tissues are bound in proteins and do not occur in the free form.

RESULTS AND DISCUSSION

The first experiment was undertaken to determine the equilibrium level of the sulfur amino acids in the regenerated wound tissues. A comparison was made with normal unwounded skin tissue. In Fig. 1 is shown the methionine, cystine and nitrogen content of wound tissue from rats fed the basal diet, altered to contain 6% casein. At about the 23rd day after wounding, the methionine and cystine content of the regenerating tissue reaches a maximum and then levels off. These levels of methionine and cystine are appreciably higher than is found in the normal skin tissue. Thus, in the wound tissue, at the maximal level, the methionine content was about 6.3 mg. and the cystine content about 8.2 mg. per gram of wet tissue. In the normal tissue, there was found 4.6 mg. and 3.0 mg. per gram of wet tissue for methionine and cystine, respectively. By the 20th day after wounding, the nitrogen content of the wound tissue was at its highest level (37.2 ± 1.8 mg. per g.) as compared to the nitrogen content of normal tissue (40.0 ± 2.1 mg. per g.). The results of the analyses for nitrogen are in essential agreement with those reported previously (9).

It is obvious that the newly synthesized proteins in the wound tissue are quite different from those which are being replaced. Further, at least two distinct types of protein are being synthesized in the wound tissue which are distinguishable by their amino acid content. During the early stages, the main type of protein being synthesized contains a larger proportion of methionine than cystine. Later on, proteins containing a higher percentage of cystine than methionine are produced in the wound tissue.

Upon reaching the maximal level, the sulfur amino acid content of the wound tissue appears to remain constant. Even 31 days after wounding, there is no indication that the level of these amino acids will fall to that found in the normal skin tissue. It should be pointed out that, after 31 days, the difficulty of visually distinguishing the regenerated from the normal unwounded tissue is so great

that it is not feasible to carry the experiment further. The possibility exists, of course, that at a later time, the sulfur amino acid content of the wound tissue may be altered.

The differences in the metabolism of the sulfur amino acids in wounded and normal animals were studied with the use of S^{35} labeled DL-cystine and DL-methionine. The use of these compounds permits an insight into the alterations in protein metabolism, protein turnover and mobilization caused by injury.

Two groups of 30 rats were fed the basal diet supplemented with 1 g. of cystine per kilo of diet. The rats in one group were wounded with the standard wound, and at intervals of 7, 11 and 14 days after wounding, 1/3 of the animals in each group were killed and the tissue analyzed. Five days after wounding, each rat was injected intraperitoneally with a tracer dose of DL-cystine-S^{35} (1.01×10^6 counts per minute). In Table I is shown the data on the methionine, cystine and nitrogen content of wound, skin, muscle and liver tissue from the wounded and normal rats.

The results of the analyses for regenerating wound tissue, in this table, follow the pattern plotted in Fig. 1. Although the cystine content of the skin, muscle and liver tissue remains essentially constant, there is a marked and significant decrease in the liver methionine of the wounded rats as compared with that found in the livers of the normal control rats. Undoubtedly there is a loss of cystine from the livers of the wounded rats, as is indicated by the results obtained in the following part of the experiment. However, the loss of cystine from the liver is evidentaly balanced by replacement with cystine from other sources, such as synthesis from methionine or from the diet, giving a net level of cystine which appears to remain unchanged. Similar effects were observed in wounded rats fed a protein free diet supplemented with DL-methionine. These results may be taken to mean that methionine from the liver is one of the principal tissue sources for the sulfur amino acids of regenerating wound tissue.

In Table II is shown the activity of the cystine-S^{35} in the tissues studied. There appears to be a rapid uptak and loss of the label by the regenerating wound

tissue. Between the 7th and 10th day after wounding, there is a 70% decrease in the specific activity of the wound tissue samples. This effect may be due either to the fact that there is a rapid accretion of wound protein and wound cystine during this time (as shown in Fig. 1 and Table I), or to protein turnover in the wound tissue, or both. It seems likely that the accretion of cystine in the wound protein has the greatest influence in causing this decrease in specific activity.

There appears to be a marked loss of S^{35} activity in the other tissues as well. Since the level of cystine in these tissues remains almost constant, this decrease in activity must be attributed to protein turnover and label dilution by the dietary cystine. The differences in the loss of S^{35} activity between the liver tissue of the normal and wounded rats are statististically significant. It would then follow that wounding has the effect of stimulating sulfur metabolism.

Another experiment was run on two similar groups of rats which were maintained on the basal diet over the whole experimental period. These animals were given 5.90×10^5 counts per minute of DL-methionine-S^{35}, intraperitoneally. The other details of the experimental procedure were generally the same as in the previously described experiment, except that the DL-methionine-S^{35} was given four days after wounding and the animals were sacrificed on the 5th, 9th and 13th days after the wound was made. The results of the analysis of the tissues were found to be very similar to those recorded in Table I. The activity of the various tissues was also determined after fractionation of the S^{35} into methionine and cystine fractions. The S^{35} activity of both fractions from the samples of skin and muscle tissue in the wounded and normal rats showed parallel rates of decrease.

In Table III is presented the data on the activity of the fractions of S^{35} from liver and regenerating wound tissue. It is to be expected that initially the methionine activity should be higher than that associated with the cystine fraction in all the tisssues, since methionine-S^{35} was administered. There appears to be a marked and relatively rapid loss of activity in the methionine fraction of the wound tissue. On the other hand, the cystine-S^{35} activity of the wound tissue

remained almost constant throughout the experimental period, although the specific activity was decreasing. This may be explained by the fact that the cystine-S^{35} which is lost from the wound protein, due to protein turnover, is being approximately replaced by labeled cystine formed from the label in the methionine released by other tissues. This acquisition of S^{35} in the form of cystine could hardly be expected to keep up with the deposition of non-labeled cystine in the wound protein, resulting in a constantly decreasing specific activity.

The uptake of methionine-S^{35} and its loss by the livers of the wounded rats in this experiment was significantly greater than that which was taken up and lost by the livers of the normal animals. This further supports the contention that there is an increased rate of sulfur metabolism in wounded rats as compared to that found in the normal rats. This increased sulfur metabolism in the wounded animals would probably result in a greater loss and a greater redeposition of cystine-S^{35} in the liver than might be expected to be found in the livers of normal rats. The summation of these effects would be expected to give the appearance of a similar rate of loss of cystine-S^{35} activity in the livers of both groups of animals

SUMMARY

During the early stage of wound tissue regeneration in rats, the methionine content of wound proteins is greater than the cystine content. Later the content of cystine becomes very much higher than that of methionine. This has been taken to mean that two different types of proteins are synthesized by the regenerating wound tissue. By the use of S^{35} labeled methionine and cystine, it was shown that sulfur metabolism is stimulated during wound tissue regeneration and that the liver methionine supplies a large part of the cystine required by the regenerating wound tissue.

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TABLE I

METHIONINE, CYSTINE AND NITROGEN CONTENT OF TISSUES IN WOUNDED AND NORMAL RATS

Days After Wounding	WOUNDED			NORMAL		
	Met mg/g	Cys mg/g	N mg/g	Met mg/g	Cys mg/g	N mg/g
			Wound Tissue			
7	3.8±0.5	2.8±0.3	19.0±0.9			
11	4.1±0.3	5.1±0.4	23.1±1.3			
14	5.5±0.3	7.3±0.2	28.2±1.4			
			Skin			
7	4.3±0.2	2.9±0.1	41.7±1.9	4.7±0.4	3.0±0.3	41.2±1.1
11	3.9±0.2	2.7±0.4	41.3±1.2	4.6±0.4	2.7±0.3	41.3±1.4
14	4.3±0.2	2.8±0.2	41.9±1.1	4.4±0.4	2.9±0.3	40.9±2.6
			Muscle			
7	6.9±0.5	3.7±0.2	36.1±1.3	7.3±0.7	3.9±0.3	37.4±1.7
11	6.6±0.3	3.7±0.3	36.4±1.0	6.6±0.5	3.9±0.5	35.7±1.4
14	6.3±0.5	3.7±0.1	35.3±1.5	6.5±0.5	3.8±0.4	37.6±1.2
			Liver			
7	6.5±0.6	4.8±0.3	33.7±1.0	6.5±0.4	4.5±0.4	33.6±0.8
11	5.1±0.4	4.4±0.3	32.8±1.0	6.5±0.6	4.7±0.2	33.6±1.1
14	4.6±0.2	4.5±0.3	28.8±1.3	6.1±0.5	4.7±0.4	32.7±1.6

All values are in terms of mg. per gram of wet tissue.

Each value represents replicate determinations from the tissue of 10 animals.

TABLE II

S^{35} ACTIVITY OF TISSUES IN WOUNDED AND NORMAL RATS AFTER THE ADMINISTRATION OF CYSTINE-S^{35}

Days After Wounding	Wound	Skin	Muscle	Liver
7	5475 ± 125	3715 ± 135	1540 ± 75	7650 ± 135
11	4560 ± 80	2060 ± 95	1510 ± 100	4695 ± 130
14	4350 ± 80	1775 ± 85	1470 ± 85	3445 ± 75
7		2970 ± 125	1970 ± 90	7805 ± 115
11		2585 ± 100	1715 ± 65	5965 ± 120
14		1740 ± 110	1545 ± 50	4315 ± 140

Calculated in counts/minute/gram of wet tissue and corrected for decay.

Each value represents duplicate determinations of the tissue of 10 rats.

TABLE III

DISTRIBUTION AND SPECIFIC ACTIVITY OF METHIONINE-S^{35} AND CYSTINE-S^{35} IN WOUNDED AND NORMAL RATS AFTER DL-METHIONINE-S^{35} ADMINISTRATION

Days After Wounding	WOUND TISSUE		LIVER	
	Activity*	Sp. Act.**	Activity*	Sp. Act.**
		Methionine-S^{35}		
5	3630 ± 110	5.30	5350 ± 200	3.15
9	2420 ± 85	2.82	4340 ± 175	2.83
13	1375 ± 60	1.35	3470 ± 110	2.62
5			4750 ± 90	2.82
9			4350 ± 90	2.72
13			3940 ± 85	2.57
		Cystine-S^{35}		
5	2630 ± 95	4.10	5000 ± 210	4.46
9	2430 ± 110	2.40	3140 ± 160	2.76
13	2480 ± 75	1.83	2980 ± 190	2.61
5			5020 ± 175	4.40
9			3060 ± 150	2.85
13			3010 ± 110	2.84

*Calculated in counts/minute/gram of wet tissue and corrected for decay.

**Sp. Act. = $\frac{\text{Counts/Minute/Gram Tissue}}{\text{mg. Cystine or Methionine S/Gm Tissue}} \times 10^{-3}$

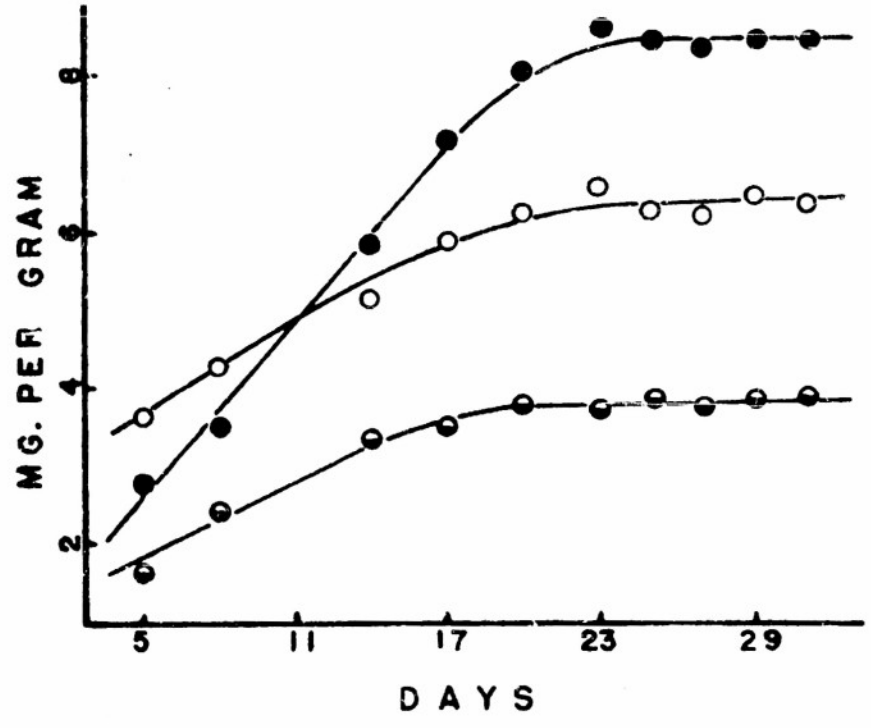

FIG. 1

Fig. 1. The concentration of cystine + cysteine, nitrogen and methionine in regenerating wound tissue plotted against time after wounding. Methionine and cystine + cysteine are plotted on the basis of mg. per gram of wet tissue. Nitrogen values are in terms of mg. $\times 10^{-1}$ per gram of wet tissue. Solid circles = cystine + cysteine; open circles = methionine; half-solid circles = nitrogen.

EXCRETION OF SULFUR DURING THE HEALING OF EXPERIMENTAL WOUNDS*

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The sulfur amino acids, methionine and cystine, appear to be unique in their ability to accelerate the rate of healing of experimental wounds to the same extent, when present in the diet in equivalent amounts (1,2,3). Earlier experiments have shown that the healing of wounds is dependant on the availability of the sulfur amino acids, rather than on the total protein nitrogen available in the diet (4,5). Since there is essentially no conversion of cystine to methionine in the organism, it may be considered that cystine is the limiting amino acid in the healing process. The role of methionine in the healing of wounds cannot be discounted, however. The methionine of the tissues has been shown to have at least two important functions in the regeneration of tissue in wounds (6); it is the primary source of cystine required for the synthesis of wound proteins; it is required per se as an integral part of the wound protein.

Since the availability of the sulfur amino acids has such a marked effect on the replacement of protein in regenerating wound tissue, it might be expected that the normal equilibria of the reactions involving these amino acids would be disturbed after wounding. This situation should be reflected in an altered metabolism of sulfur during wound tissue regeneration. In this paper, the results of a comparison of the sulfur metabolism, as indicated by sulfur excretion, in normal and wounded rats will be described.

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EXPERIMENTAL. In the following experiments, female albino rats weighing 200 ± 20 g were used. The animals were maintained on a basal protein-free diet for 5 days prior to wounding. The basal diet consisted of 83 g sucrose, 10 g lard, 2 g corn oil, and 5 g salt mixture (7). The basal diet also contained a vitamin supplement, whose constituents and proportions have been previously enumerated (2). The animals were offered 8 g of diet per day, which was completely consumed before the next daily feeding period, so that the dietary intake of all the animals was isocaloric. Distilled water was permitted ad libitum.

After 5 days, the hair on the back of the neck and shoulders was removed and an area of skin 4 cm in diameter, was incised down to the fascia. The tissue was then removed with the underlying fascia. Control rats were treated in a similar manner except that no wounds were made. The rats were housed in metabolism cages and urine samples collected every 24 hours after wounding. The samples of urine were stored under toluene in a tightly stoppered bottle at 5° C. until the analyses could be performed. The urine samples were analyzed for nitrogen (microkjeldahl) and total sulfur (8,9). After hydrolysis of an aliquot of the urine for 30 minutes in 6 N HCl on a boiling water bath, the total sulfate was determined as the barium salt (8).

In the experiment in which tracer doses of S^{35} labeled DL-methionine and DL-cystine were administered to the rats, the total S^{35} activity of the urine, as well as the total sulfate activity, isolated as indicated above, was determined. The S^{35} activity was measured using a Tracerlab Autoscalar and a Geiger-Muller tube having a window thickness of 1.4 mg per cm^2.

RESULTS AND DISCUSSION. Studies on the excretion of nitrogen and sulfur were made in two groups of 30 rats kept on a basal protein-free diet. The animals in one group were wounded, while the second group of animals served as controls. The excretion of nitrogen during the first two weeks after wounding is shown in Fig. 1. In accordance with the results of previously published experi-

ments (5,10,11), the wounded rats were found to excrete considerably more nitrogen than the control rats. In Fig. 2 is plotted the total sulfur excretion against time after wounding. It can be seen that the wounded and control rats excreted approximately the same amount of sulfur. The data in these two graphs are indicative of the fact that there is an actual retention of sulfur by the wounded rats. It would be expected that the breakdown and metabolism of tissue protein should result in a relatively constant ratio of nitrogen and sulfur excretion, regardless of the amount of nitrogen excreted. However, in this experiment, the ratio of nitrogen to sulfur excreted by the wounded rats is greater than by the controls, indicating a relative net retention of sulfur by the wounded animals.

Fig. 3 shows the excretion of sulfate by the wounded and normal rats. The wounded rats appear to excrete about 40% more sulfate than do the normal control animals. Since sulfate is the end product of sulfur metabolism, this may be taker to mean that the metabolism of the sulfur amino acids is increased in wounded rats as compared to normal ones.

The overall metabolic path of the sulfur in the sulfur-containing amino acids can be abbreviated into the following reactions:-

$$\text{Methionine} \longrightarrow \text{Cystine} \longrightarrow \text{Sulfate} \qquad (1)$$

The importance of cystine during the regeneration of wound tissue led us to study the conversions in the above equation. This was done by following the excretion of S^{35} after the administration of S^{35} labeled methionine and cystine.

The experiment was carried out on 4 groups of 15 rats, maintained on the basal diet. Two groups of rats were wounded as indicated before. Four days after wounding, one group of wounded and one group of unwounded control rats were injected intraperitoneally with DL-methionine-S^{35} (5.90×10^5 counts per minute per rat). The other two groups received DL-cystine-S^{35} (6.06×10^5 counts per minute per rat) at the same time. The total S^{35} activity in the urine and the S^{35} activity associated with the sulfate in the urine was determined daily thereafter.

As might be expected from the results in the previous experiment, the total daily excretion of S^{35} was essentially the same for the wounded and control animals. However, the level of S^{35} excretion was higher in those rats receiving the labeled cystine as compared to those receiving the labeled methionine. This is probably due to the fact that the methionine-S^{35} can be utilized either as such or as cystine-S^{35} for protein synthesis and turnover; on the other hand, cystine-S^{35} is utilizable for incorporation into protein only in the unmetabolized state. Thus, there are two forms in which the S^{35} from the methionine-S^{35} can be deposited and retained in the tissues, while that from the cystine-S^{35} has only one. This would be expected to result in a greater retention of injected methionine-S^{35} than cystine-S^{35}, and a concommitant greater excretion of injected cystine-S^{35} than methionine-S^{35}.

The data for the excretion of sulfate-S^{35} in this experiment is shown in Table I. It can be seen that the wounded rats which received the methionine-S^{35} excreted more sulfate-S^{35} than did the controls. This must mean that either or both of the series of reactions represented in equation (1) are accelerated in the wounded rats. Data presented in another report (12) indicates that the rate of conversion of methionine to cystine is greater in wounded than in normal rats. In the animals receiving the cystine-S^{35}, it was again observed that the wounded ones excreted more sulfate-S^{35} than did the controls. This can only be interpreted to mean that there is a higher rate of oxidation of cystine in the wounded rats during the regeneration of wound tissue. It may then be concluded that during the process of regeneration of wound tissue, the metabolism of the sulfur amino acids is stimulated.

SUMMARY. The effect of wounding on the metabolism of the sulfur amino acids was studied by following the excretion of sulfur in normal and wounded rats. After wounding, there is a relative retention of sulfur, even though a

greater amount of nitrogen than normal is excreted. Studies with S^{35} labeled cystine and methionine indicated that the metabolism of these amino acids is greater in wounded than in normal animals.

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TABLE I

EXCRETION OF LABELED SULFATE BY WOUNDED AND NORMAL RATS AFTER THE ADMINISTRATION OF DL-METHIONINE-S^{35} AND DL-CYSTINE-S^{35}

Days After Wounding	METHIONINE*		CYSTINE*	
	Normal	Wounded	Normal	Wounded
5	4680	4840	5300	5300
6	2940	4490	5110	5600
7	2710	4100	4600	7450
8	2290	2500	3900	4500
9	1950	2750	2530	4550
10	1880	2670	2760	4550
11	1785	2230	2630	4430

*Counts per minute per 24 hour sample, corrected for decay to the time of injection and for difference in dose of the methionine-S^{35} and cystine-S^{35}. The labeled amino acids were injected on the 4th day after wounding.

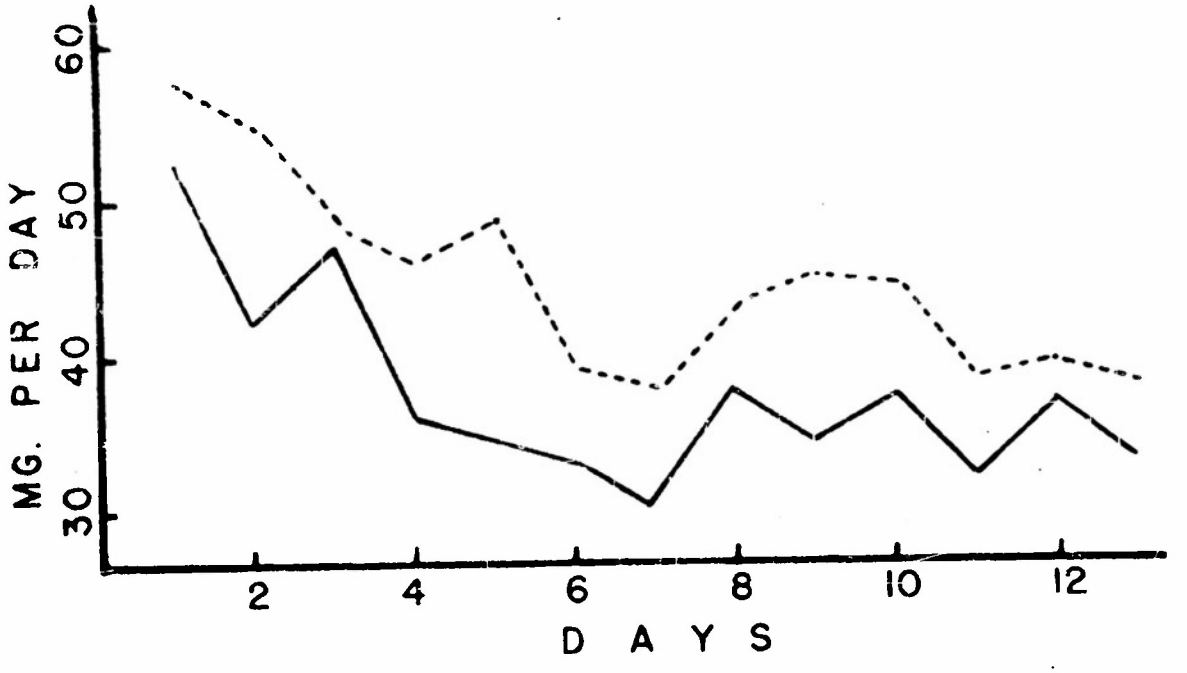

Fig. 1

Fig. 1. The excretion of nitrogen by normal and wounded rats plotted in mg. per day against days after wounding. Solid line = control rats; Broken line = wounded rats.

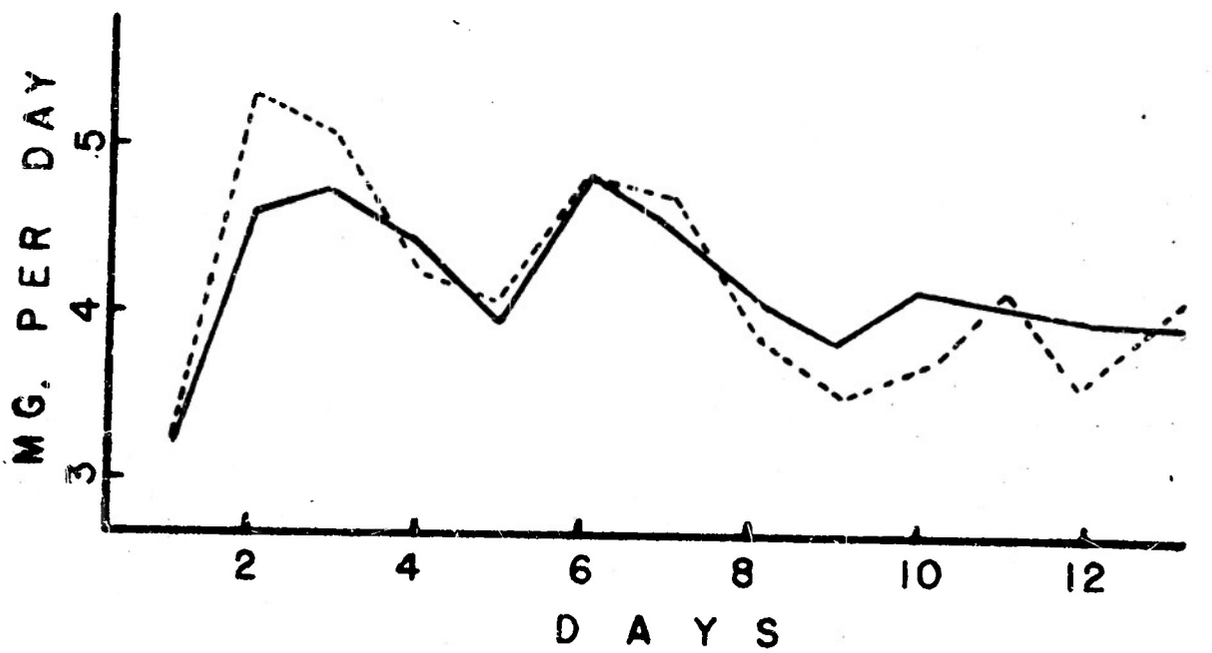

Fig. 2

Fig. 2. The excretion of sulfur by normal and wounded rats plotted in mg. per day against days after wounding. Solid line = control rats; Broken line = wounded rats.

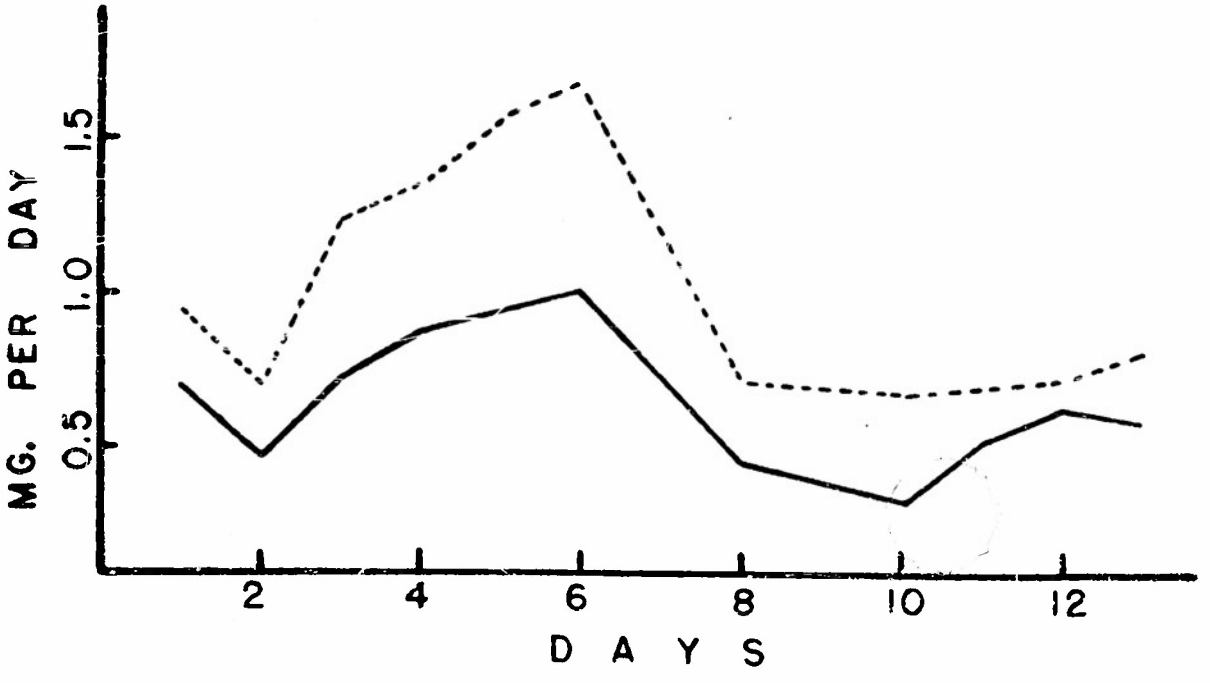

Fig. 3

Fig. 3. The excretion of sulfate sulfur by wounded and normal rats plotted in mg. per day against days after wounding. Solid line = control rats; Broken line = wounded rats.